THE CHANGE IN MEMBRANE FLUIDITY OF NEUROBLASTOMA × GLIOMA HYBRID CELLS UPON CELL DIFFERENTIATION

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1. Introduction

The neuronal cells exhibit morphological changes upon cell differentiation such as neurite outgrowth. The neurites have special rôles; transmission of action potential, recognition of target cells and the formation of synapses. Therefore it is expected that the membrane properties of neurites may differ from that of cell soma.

In the study of membrane properties of neuronal cells, cell lines are often used as model systems due to advantages such as the easiness for obtaining large number of cells, cell homogeneity and the easiness in controlling environments. Neuroblastoma × glioma hybrid cell line, NG108-15, can be regarded as one of the best models for neuronal cells. NG108-15 cells synthesize and release acetylcholine [1], synthesize an enkephalin-like peptide [2], form functional synapses with skeletal muscle cells in culture [1,3] synthesize the promoting factor of acetylcholine receptor aggregation in muscle cells [4], and have many kinds of receptors [5–8]. Therefore, it is worthwhile to study membrane properties of NG108-15 cells in order to investigate the mechanisms of the differentiation of neuronal cells.

Here, we report the changes in translational motions of fluorescent analog of fatty acid incorporated into NG108-15 cells upon cell differentiation.

2. Materials and methods

2.1. *Cell line*

Neuroblastoma × glioma hybrid cells, NG108-15, were plated onto cover slips in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 1×10^{-4} M hypoxantine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine (HAT medium) and 5% newborn calf serum. The cells were differentiated by culturing in the above medium supplemented with 1 mM N^6, $O^{2'}$-dibutyryladenosine 3′:5′-cyclic mononucleotide (Bt_2CAMP) for 3 days.

Abbreviations: F18, 5-(octadecylthiocalbamoylamino)fluorescein; Bt_2CAMP, N^6, $O^{2'}$-dibutyryladenosine 3′:5′-cyclic mononucleotide; FPR, fluorescence photobleaching recovery

2.2. *Fluorescent probe*

The fluorescent analog of fatty acid, F18, was synthesized as in [9].

2.3. *Labelling of the cells*

Cells grown on cover slips were washed 2 times with Hepes saline buffer (150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 5 mM Hepes–NaOH (pH 7.2)), and incubated in the same buffer solution containing 15 μM F18 for 3 min at 37°C. Then cells were washed 3 times with the same buffer solution, and placed in a chamber filled with the buffer solution.

2.4. *Fluorescence photobleaching recovery measurements*

Fluorescence photobleaching recovery (FPR) measurements were performed with almost identical equipment to that in [10,11]. The optical alignment is shown in fig.1. The spacial profile of incident light was controlled to be flat (mixture of TEM_{00} and TEM_{01} mode). The bleached area was selected by inserting a slit or a pinhole (SL). A quarter wavelength plate (Q) inserted converts the linearly polarized light into circularly polarized one and it reduces the effect of rotational motion.

The lateral diffusion coefficients were obtained from following equations:

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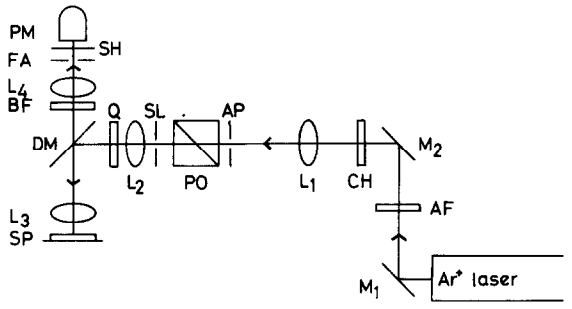

Fig.1. Optical alignment for FPR measurement: Ar^+ laser (coherent radiation CR4); M_1, M_2, mirrors; AF, laser intensity attenuation filter; CH, chopper; L_1, L_2, L_3, L_4, lenses; AP, aperture; PO, glan laser polarizer (Karl Lambrecht); SL, slit or pinhole; Q, a quarter wavelength plate (Polaroid); DM, dichroic mirror; SP, specimen; BF, barrier filter; FA, field selecting aperture; SH, electronic shutter (copal); PM, photomultiplier (Hamamatsu TV R464S). Optical microscope used was Nikon apophot fluorescence microscope. The line of 4880 Å was used for experiments. Attenuation filter (AF) was removed during bleaching and was reinserted during recovery measurements. Attenuation was ~1/4000. Incidient laser light was linearly polarized by PO and then circularly polarized by Q. Spectral band width of the light from Ar^+ laser was 10 GHz which corresponds to the coherent time of <0.1 ns. Bleaching time (0.22 s) being much longer than this coherent time, the circularly polarized light irradiated the Specimen (SP) as a randomly polarized light, and this reduced the effect of rotational motion on the measurement of lateral motion. A slit and a pinhole inserted created 6.8 μm × 56 μm and 5.1 μm diam. of bleached areas, respectively, when a 100 × objective lens was used.

$$D = 0.22\omega^2/\tau_{1/2} \quad \text{for disk (2-dimensional) [12]}$$

$$D = 0.23d^2/\tau_{1/2} \quad \text{for rectangular slit (1-dimensional)}$$

where ω is the radius of bleached area, $\tau_{1/2}$ is the half-recovery time and d is the slit width. The fraction of recovery, f, was defined as:

f = (recovered fluorescence intensity)/(reduced fluorescence intensity by bleaching)

3. Results

Fig.2 illustrates the phase contrast and fluorescence microscopic images of NG108-15 labelled with F18. It is clear that F18 labels specifically cell surfaces judging from the ring-like pattern. This fluorescence pattern was stable for >1 h at 24°C. It can be concluded, therefore, that F18 is a suitable probe for cell membranes.

The effect of bleaching pattern on apparent lateral diffusion coefficients was studied. Since neurites of neuronal cells are often thin (diameter less than several μm), the lateral motion of probes are pseudo 1-dimensional, and cannot be analyzed as 2-dimensional motion. If we bleach the cells with a rectangular shape slit, the motion of probes are regarded as 1-dimensional. The comparison of disk and rectangular slit bleachings are shown in table 1.

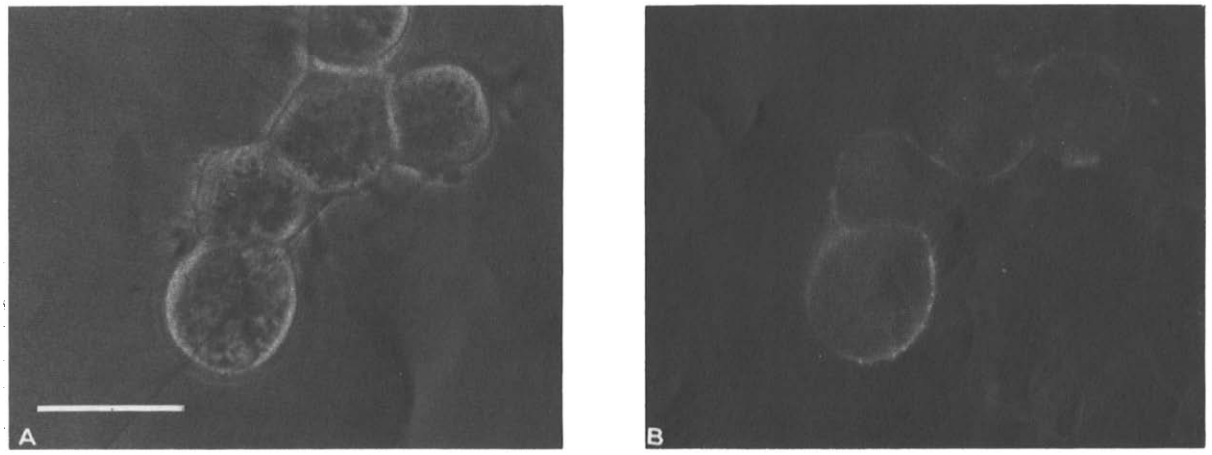

Fig.2. Phase contrast and fluorescence microscopic images of NG108-15 labelled with F18: (A) phase contrast image; (B) fluorescence image. The cells were grown in HAT medium supplemented with 5% newborn calf serum for 4 days. Bar indicates 30 μm.

Table 1
Effect of bleaching patterns on apparent lateral diffusion coefficients, D, of F18 labelled to NG108-15

	Bleaching pattern	D at 24°C ($\times 10^{-8}$ cm²/s)	No. cells
Soma	Disk	0.25 ± 0.10	(5)
	Rectangular slit	0.28 ± 0.11	(5)
Neurite	Disk	0.38 ± 0.10	(3)
	Rectangular slit	0.63 ± 0.12	(3)

Cells were grown in HAT medium supplemented with 5% newborn calf serum for 4 days and were differentiated by further supplement of 1 mM Bt_2CAMP for 3 days. Labelling condition was as in fig.2. The bleaching by each pattern was performed at the same position of the cell. Errors are standard deviations.

In soma, where the motion is 2-dimensional, the effect of the bleaching pattern was negligible. However, in neurite, the apparent diffusion coefficients obtained by the disk pattern bleaching were considerably smaller than that obtained by the rectangular slit pattern bleaching. It can be concluded from table 1, that in observing the lateral motion of probes in fibrous portion, the rectangular slit pattern bleaching is necessary and bleaching with point symmetrical pattern (Gaussian or disk pattern) leads to erroneous result.

The effect of cell differentiation on the lateral diffusion of F18 is shown in table 2.

Upon cell differentiation, the lateral diffusion coefficient of F18 became small in cell soma, while in neurites, it became large. The mobile fraction of F18 was reduced after differentiation in cell soma. According to these results, it can be concluded that upon cell differentiation of NG108-15, the heterogeneity of membrane property is generated; cell membranes of neurites become fluid and those of cell soma become rigid.

Other proteinous probes, concanavalin-A, succinilated concanavalin A, *Ricinus communis* agglutinin and cationized ferritin conjugated with fluorescein isothiocyanate showed very reduced motion (lateral diffusion coefficient $<10^{-11}$ cm²/s) in every case.

4. Discussion

As shown in fig.2, the fluorescent probe F18 was proved to be a very suitable surface labelling probe. F18 could also successfully label the cell surface of chick dorsal root ganglional neuronal cells [13], myogenic cell line, L-6 [14], and fibroblast cells in culture and could detect changes in membrane fluidity. Dead cells or cell debris were homogeneously labelled. One can, therefore, identify vital cells from dead cells by observing fluorescence images. Internalization of F18 was negligible within 1 h at 24°C.

The effect of bleaching pattern was compared. The fiber-like shape of neurites gave rise to difficulty in the analysis of FPR results when the point symmetrical pattern bleaching was employed. Employment of the rectangular slit pattern bleaching eliminated this difficulty, and the lateral motion in neurites and in cell soma could be directly compared.

Here, we report that the membrane property changed upon cell differentiation, and also the membrane property of neurites and of cell soma were different. We had suggested that in another neuroblastoma cells, N18 (a parent cell line of NG108-15), the membrane fluidity decreased upon cell differentiation [15]. However, in [15] the fluidity observed was the average of those of neurites and of cell soma. No information from localized portion of the cells was

Table 2
The cell differentiation-dependent changes in apparent lateral diffusion coefficients, D, and in mobile fractions, f, of F18 labelled to NG108-15

		D at 24°C ($\times 10^{-8}$ cm²/s)	f	No. cells
Undifferentiated	–	0.46 ± 0.16	0.90 ± 0.11	(15)
Differentiated	Soma	0.26 ± 0.07	0.67 ± 0.12	(7)
	Neurites	0.60 ± 0.16	0.90 ± 0.09	(6)

Undifferentiated cells were grown in HAT medium for 7 days. Differentiated cells were obtained as shown in Table 1. Errors are standard deviations

shown. Present data suggests that the investigation of localized parts of cells gives more detailed information.

Another neuroblastoma cell (Neuro-2A) exhibited enhanced fluidity of neurite membranes upon cell differentiation [16]. Taking into consideration the results in [16] and here, it is plausible that it is the general property that:

(1) Upon cell differentiation of neuronal cells, the local change in membrane fluidity becomes significant;
(2) Membranes of neurites become more fluid than those of cell soma.

Many physiological and biochemical changes accompanying cell differentiation of neuronal cells have been reported [17–19]. Unfortunately, however, little has been reported about the differences between neurites and cell soma. There is much possibility that the changes in fluidity measured here are closely correlated with physiological and biological changes of neuronal cells. Optical detection of membrane potential can detect local electrical membrane property [20]. Combination of this measurement with FPR will be a powerful tool to investigate detailed membrane property of neuronal cells.

5. Conclusion

The lateral motions of fluorescent fatty acid analog, 5-(octadecylthiocalbamoylamino)fluorescein, F18 in cell membranes of neuroblastoma × glioma hybrid cell line, NG108-15, were measured. F18 specifically labelled the cell surface. The lateral motions of F18 were reduced in cell soma while these were increased in neurites upon cell differentiation. At the same time, the immobile fractions of F18 increased in cell soma.

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